69. (Once Amended) The kit according to claim 68, wherein said anti-antibody antibody is specific for human immunoglobulins.

70. (Once Amended) The kit according to claim 68, wherein said anti-antibody antibody is directly or indirectly labelled, either using a labelling substance, or by an enzyme which emits a labelling signal through the transformation of its substrate.--

REMARKS

Entry of the foregoing and favorable reconsideration and reexamination of the subject application, as amended, pursuant to and consistent with 37 C.F.R. Section 1.112, and in light of the remarks which follow, are respectfully requested.

By the present amendment, the specification has been amended to reflect the SEQ ID numbers and the international deposit registration numbers of hybridomas producing monoclonal antibodies 3921E4 and 4057D2, as well as to clarify the legends of the Figures. Claims 56 to 64, 66, 67, 69, and 70 have been amended to better U.S. format. Claims 71 to 81 have been cancelled since they are drawn to a non-elected invention. Applicants reserve their rights to file a divisional application directed to the canceled subject matter. Applicants submit that no new matter has been added by the present amendment.

Turning now to the Official Action, Applicants have canceled the claims drawn to the non-elected invention.

As far as the drawings are concerned, the SEQ ID numbers have been inserted appropriately into the specification and the Brief Description of the Figures has been amended to set forth Figures 1A, 1B, 5A, and 5B.

The Examiner has objected to the specification due to several informalities. Most of these informalities have now been rendered moot by amendment. As far as the objection to immunitary serum at page 11 is concerned, this objection is respectfully traversed.

By immunitary serum is meant a serum containing anti-HBHA antibodies. These antibodies can be either monoclonal, as exemplified by the 3921E4 antibody, or polyclonal, as exemplified by the anti-HBHA rat polyclonal antibodies. Both of these antibodies are described in the specification. A skilled artisan would realize that immunitary serum encompasses both of these antibodies.

Therefore, in view of the above, withdrawal of this objection is respectfully requested.

Claims 57, 59 and 62 have been objected to since the term "recognized" is misspelled in the latter two claims and the sequence identifier is not inserted in Claim 57. These claims have been amended accordingly rendering this objection now moot.

The Examiner deems that the specification lacks deposit information for monoclonal antibodies 4057 D2 and 3921 E4. The specification has been amended to recite international deposit registration numbers of the hybridomas producing said monoclonal antibodies, together with the depository name and full address.

Applicants are enclosing herewith a copy of a facsimile letter, together with a certified English translation thereof, from Mr. Georges Wagener, responsible at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, Paris, France) for the exercise of the duties of an International Depository Authority under the Budapest Treaty, said facsimile letter confirming that the hybridomas producing monoclonal antibodies 4057 D2 and 3921 E4 were submitted to the CNCM for international deposit registration.

Deposit receipts, as well as a Declaration concerning the deposit of microorganisms will be submitted as soon as a Notice of Acceptance of said deposits is emitted by the CNCM.

Therefore, in view of the above, withdrawal of this objection is respectfully requested.

Claims 56 to 59 and 65 have been rejected under 35 U.S.C. §101 since the claimed invention was directed to non-statutory subject matter. Claim 56 has been amended to insert the word "isolated" which should render this rejection now moot.

Claims 59 and 62 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. These claims have been amended to include deposit registration numbers of the hybridomas producing monoclonal antibodies 3921 E4 and 4057 D2, as well as the International Depository Authority under the Budapest Treaty name.

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 56 to 70 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. This rejection has been rendered moot by amendment to these claims.

More specifically, Claims 56, 60, 63 and 66 have been amended to recite the SEQ ID numbers. The C-terminal portion of the peptide sequence has been further clarified and the claims have been amended to delete the term "being involved."

Therefore, in view of the amendment, withdrawal of this rejection is respectfully requested.

Claims 56 to 67 have been rejected under 35 U.S.C. § 102 (b) as being anticipated by Menozzi et al.

Applicants are enclosing a certified copy of the priority document. The priority date for the present invention is May 17, 1996. The publication of Menozzi et al was presented at a Congress of May 21 to 25, 1995. Therefore, Menozzi et al is not prior art under 35 U.S.C. § 102 (b).

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

From the foregoing, favorable action in the form of a Notice of Allowance is respectfully requested and such action is earnestly solicited.

This application is submitted to be in condition for allowance and a Notice to that effect is requested.

Respectfully submitted,

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By Mary J. Wilson
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Marked-up version of the specification

At page 3, first full paragraph, please delete this paragraph and substitute the following paragraph:

In a preferred embodiment of the present invention, the peptide sequence is characterized in that it comprises the sequence corresponding to the sequence shown in Figure 10 (SEQ ID No. 19), or any variant of that sequence which enables mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of the sequence of Figure 10 (SEQ ID No. 19).

At page 3, paragraph 3, please delete this paragraph and substitute the following paragraph:

Preferably, the invention more particularly provides a peptide sequence comprising a region involved in interactions with sulphated glycoconjugates and in heparin binding. This peptide sequence is as follows:

KKAAPAKKAAPAKKAAPAKKAAAKKAPAKKAAAKKVTQK (SEQ ID No. 1).

At page 5, second paragraph, please delete this paragraph and substitute the following paragraph:

The present invention will now be described with reference to the following Figures:

- Figure 1A is a graph illustrating an adhesion test carried out with CHO in the presence of increasing concentrations of D(+)galactose (black circles) or heparin (white circles) from pork intestinal mucosa.
- Figure 1B shows the effect of sulphated and non sulphated glucides on mycobacterial adhesion to CHO cells and to macrophages;
- Figure 2 shows data demonstrating purification of a heparin binding protein of *M. bovis* BCG;
- Figure 3 shows a comparison of the heparin binding protein of *M. bovis* with the antigen 85 complex;

- Figure 4 shows the effect of sulphated glucides and non sulphated glucides on haemagglutinination induced by HBHA;
- [Figure 5 shows the inhibition of adhesion of BCG to CHO cells by anti-HBHA antibodies;]
  Figure 5A is a graph illustrating the inhibition of adhesion of BCG to CHO cells by anti-HBHA monoclonal antibodies 3921E4;
- Figure 5B is a graph illustrating the inhibition of adhesion of BCG to CHO cells by anti-HBHA polyclonal antiserum;
- Figure 6 shows immunoblot analyses carried out with tuberculous anti-sera;
- Figure 7 shows the nucleotide sequence (SEQ ID No. 17) and the amino acid sequence (SEQ ID Nos. 17 and 18) of a fragment of HBHA deduced from a PCR fragment of chromosomal BCG DNA ;
- Figure 8 shows a Southern blot analysis of chromosomal BCG DNA;
- Figure 9 shows the sequencing strategy for the gene coding for HBHA;
- Figure 10 shows the DNA sequence of the BCG gene coding for HBHA (SEQ ID No. 19);
- Figure 11 shows a polyacrylamide gel electrophoresis and immunoblot analysis of the expression of HBHA in *E. coli*.

At page 6, paragraph 3, please delete this paragraph and substitute the following paragraph:

The day before the adhesion test, the wells of 24-well tissue culture plates (Nunclon, Nunc, Denmark) were inoculated with 10^5 extemporaneously cultivated cells from the ovaries of Chinese hamsters (CHO) (see [Figures] Figure 1A, and Figure 1B, [grey] open bars) or J774A.1 macrophages (ATCC TIB67) (see Figure 1B, [black] speckled bars) taken up into suspension in 2 ml of RPMI with an added 10% (v/v) of decomplemented foetal calf serum (RPMI-FCS). Just before the test, the cells were washed three times with 2 ml of RPMI, and 1 ml of the mycobacterial suspension in RPMI was added to each well to obtain an infection multiplicity of 10 bacteria per eukaryote cell.

At page 9, fourth paragraph, please delete this paragraph and substitute the following paragraph:

The capacity of bacterial adhesins to agglutinate [red globules] erythrocytes is often used as a model to study microbial binding such as that of lectins to the receptor in eukaryotic cells. The inventors thus tested the purified heparin binding protein for its capacity to agglutinate erythrocytes.

At page 10, first full paragraph, please delete this paragraph and substitute the following paragraph:

Less than 0.1 µg of purified protein was able to induce haemagglutinination of rabbit erythrocytes, but not of human, sheep, goose or chicken erythrocytes (data not shown). For this reason, the inventors designated this protein as being [he] the heparin binding haemagglutinin (HBHA). Haemagglutinination induced by HBHA was inhibited by heparin or by dextran sulphate, but not by dextran (see Figure 4), which is similar to the results obtained in the tests for adhesion of mycobacteria to CHO cells. The haemagglutinination activity was heat sensitive in that incubation of the protein for 60 minutes at 80°C destroyed haemagglutinination.

At page 11, first full paragraph, please delete this paragraph and substitute the following paragraph:

The BCG was labelled with [6-^{3}H]uracil as described above. Approximately 4 x 10^{6} radiolabelled BCG were taken up into suspension in 1 ml of PBS and pre-incubated with the indicated volumes of ascetic liquids of monoclonal antibodies 3921E4 (14) anti-HBHA (Figure 5A), or with 250 µl of rat anti-HBHA polyclonal antiserum (immunitary serum, Figure 5B) or naïve serum (Figure 5B, control serum) for 30 minutes, at room temperature. The hybridoma producing the 3921E4 anti-HBHA monoclonal antibodies was deposited on June 25, 2002, at the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, 25 rue du Docteur Roux, Paris 15, France) under the registration number CNCM I-2900. The bacterial suspensions were then washed three times with 2 ml of DPBS to eliminate the non

bound antibodies, then used in the CHO cell adhesion test in an infection multiplicity of 10, as described above.

At the last paragraph bridging pages 11 and 12, please delete that paragraph and substitute the following paragraph:

Purified heparin binding protein from a preparation of *M. bovis* BCG cell wall (Figure 3, track 1) or culture supernatant (track 2) was compared with purified antigen 85 complex (track 3) by immunoblot analysis (panels A, B, C) and staining with Coomassie Blue (panel D) after SDS-PAGE. Immunoblot analysis was carried out using polyclonal antibodies directed against the purified heparin binding protein (panel A), the monoclonal antibody 4057D2 (panel B) or polyclonal antibodies directed against antigen complex 85 (panel C). The hybridoma producing the 4057D2 anti-HBHA monoclonal antibody was deposited on June 25, 2002, at the Collection Nationale de Cultures de Microorganismes (see above) under the registration number CNCM I-2901. Tracks 1 and 2 of panels A, B and C contained 2 µg of purified protein, tracks 3 of panels A, B and C contained 7 µg of purified protein, and tracks 2 and 3 of panel D contained 4 µg and 15 µg of purified protein respectively. The size of labels M_r is shown in the margin.

At page 12, second paragraph, please delete this paragraph and substitute the following paragraph:

The N-terminal amino acids of the purified heparin binding proteins of H37Ra *M. tuberculosis* and of BCG were sequenced. This was accomplished by subjecting 25 µg of HBHA to polyacrylamide-SDS gel electrophoresis using a 15% polyacrylamide gel. After electrophoresis, the material was transferred to a PVDF membrane (ProBlott, ABI) by electroblotting. After staining with Coomassie blue, the band corresponding to HBHA was excised and underwent automatised Edman degradation. The first 16 amino acids were Ala-Glu-Asn-Ser-Asn-Ile-Asp-Asp-Ile-Lys-Ala-Pro-Leu-Leu-Ala-Ala (SEQ ID No. 20 from amino acid positions 2 to 17). The first 16 amino acids in the BCG heparin binding protein were also determined and proved to be identical to those of *M. tuberculosis*. A similarity search in protein databases showed that the heparin binding protein had not been identified before,

and that it thus represents a novel mycobacterial protein. Further, the first 16 amino acids did not exhibit any significant sequence similarity with other known protein sequences.

At the last paragraph bridging pages 12 and 13 please delete that paragraph and substitute the following paragraph:

To clone the gene coding for HBHA, firstly the N-terminal sequences of internal HBHA fragments were determined. To this end, purified HBHA underwent electrophoresis as described above. After electrophoresis, the protein was digested with trypsin inside the gel. The resulting peptides were then isolated by reverse phase HPLC, and then underwent Edman degradation. Four peptides enabled the sequence to be determined:

Peptide S1441:	Lys-Ala-Glu-Gly-Tyr-Leu-Glu-Ala-Ala-Thr (SEQ ID No. 2)
Peptide S1443:	Xxx-Glu-Gly-Tyr-Val-Asp-Gln-Ala-Val-Glu-Leu-Thr-Gln-Glu-Ala-Leu-Gly-Lys (SEQ ID No. 3)
Peptide S1446:	Xxx-Gln-Glu-Xxx-Leu-Pro-Glu-Xxx-Leu (SEQ ID No. 4)
Peptide S1447:	Phe-Thr-Ala-Glu-Glu-Leu-Arg (SEQ ID No. 5)

At page 13, first full paragraph, please delete this paragraph and substitute the following paragraph:

The sequence of two pairs of oligonucleotides was derived from the internal HBHA peptide sequences. The generally high G+C content in the mycobacterial DNA has led the inventors to favour G or C in the third position of the codons (wobble). The first pair of oligonucleotides originated from the S1441 peptide and had the following sequences: 5'AAG GC(G/C) GAG GG(G/C) TAC CT 3' (oligo S1441) (SEQ ID No. 6) and 5' AGG TA (G/C) CCC TC(G/C) GCC TT 3' (reverse oligo S1441) (SEQ ID No. 7). The second pair of oligonucleotides originated from the S1443 peptide and had the following sequences: 5'GAC CAG GC(G/C) GT (G/C)

GAG CT 3' (oligo S1443) (SEQ ID No. 8) and 5' AGC TC (G/C) AC(G/C) GCC TGG TC 3' (reverse oligo S1443) (SEQ ID No. 9).

At page 13, second paragraph, please delete this paragraph and substitute the following paragraph:

The chromosomal BCG DNA was extracted as described by Kremer et al [~~(23)~~](16). Polymerisation chain reactions (PCR) using 50 ng of chromosomal BCG DNA and 1 µg of either reverse S1441 and S1443, or reverse oligo S1443 and S1441, were carried out at a hybridisation temperature of 50°C with 30 PCR cycles. Only the PCR carried out with the reverse S1441 and S1443 oligonucleotides produced a specific amplified DNA fragment of approximately 150 bp. This amplified fragment was again observed when the hybridisation temperature was increased to 57°C.

At page 16, in Table I, please delete this Table and substitute the following Table:

TABLE I: Oligonucleotides used for sequencing the gene coding for HBHA.

Name of oligonucleotide	Sequence
HBHA Seq1	5'AGC CGG TAC AAC GAG CTG GTC 3' (SEQ ID No. 10)
HBHA Seq1inv	5'GAC CAG CTC GTT GTA CCG GCT 3' (SEQ ID No. 11)
HBHA Seq2	5'CAT CCA ACA CGT CGA CTC C 3' (SEQ ID No. 12)
HBHA Seq3	5'TTG ATG TCA TCA ATG TTC G 3' (SEQ ID No. 13)
HBHA Seq4	5'CGT GGA CCA GGC GGT GGA G 3'(SEQ ID No. 14)
HBHA Seq5	5'GAC GAT CAG GAG GTT TCC CCG 3'(SEQ ID No.15)
Reverse primer	5'AGC GGA TAA CAA TTT CAC ACA GGA 3' (SEQ ID No. 16)

At page 19, first full paragraph, please delete this paragraph and substitute the following paragraph:

The invention thus also concerns a recombinant peptide sequence characterized in that it enables mycobacteria to adhere to host cells. More particularly, the invention concerns a peptide sequence comprising a polypeptide of

about [~~25~~] 27 kDa recognised by the monoclonal antibody 3921E4 (14) and not recognised by the monoclonal antibody 4057D2 (14). Preferably, the recombinant peptide sequence of the invention is the expression product of a strain of *E. coli* transformed with one of the nucleotide sequences described above and coding for a peptide sequence enabling mycobacteria to adhere to host cells, more particularly a nucleotide sequence obtained from *M. bovis* BCG or *M. tuberculosis*. The invention also concerns any variation in this recombinant peptide sequence obtained by addition, substitution or deletion of one or more nucleotides such that the transformed strain produces a different peptide sequence but which possesses the property of adhering to host cells, and more particularly to epithelial cells.

MARKED-UP VERSION OF THE CLAIMS :

56. (Once Amended) An isolated proteinic mycobacterial antigen comprising 30 to 50 amino acids of a [the] C-terminal portion of SEQ ID No. 19 [the peptide sequence shown in Figure 10, and more particularly all or a portion of the last 50 amino acids, or any variant of that sequence obtained by addition, substitution or deletion of one or more amino acids, said antigen being involved in], or a variant of said 30 to 50 amino acids of said C-terminal portion of SEQ ID No. 19, or part of the last 50 amino acids in said C-terminal portion of SEQ ID No. 19, or a variant of said part of the last 50 amino acids in said C-terminal portion of SEQ ID No. 19, wherein said variant is obtained by addition, substitution or deletion of one or more amino acids and said antigen enables the adhesion of mycobacteria to the sulphated glucides of epithelial cells.

57. (Once Amended) [An] The antigen according to claim 56, wherein [characterized in that] the peptide sequence involved in the adhesion function is comprised in the following sequence:

KKAAPAKKAAPAKKAAPAKKAAAKKAPAKKAAAKKVTQK (SEQ ID No. 1)

or any portion or variant of this sequence enabling mycobacterium to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said peptide sequence.

58. (Once Amended) [An] The antigen according to claim 56, wherein said antigen is obtainable from *M. bovis* BCG or *M. tuberculosis*.

59. (Once Amended) [An] The antigen according to claim 56, [characterized in that it] wherein said proteinic mycobacterial antigen is [recognised] recognized by the monoclonal antibodies 4057 D2 (deposited at the CNCM under the number CNCM I-2901) and 3921 E4 (deposited at the CNCM under the number CNCM I-2900).

60. (Once Amended) A recombinant peptide sequence which is obtainable by expression in a host cell of a polynucleotide sequence [shown in Figure 10] of SEQ ID No. 19, and [constituting] wherein said recombinant peptide sequence is an HBHA mycobacterial antigen [involved in] enabling the adhesion of mycobacteria to the sulphated glucides of epithelial cells.

61. (Once Amended) [A] The peptide sequence according to claim 60, [characterized in that] wherein the polynucleotide sequence is obtained from *M. bovis* BCG or *M. tuberculosis*.

62. (Once Amended) [A] The peptide sequence according to claim 60, [characterized in that it] wherein said peptide sequence is [recognised] recognized by [the] a monoclonal antibody 3921 E4 (deposited at the CNCM under the number CNCM I-2900) and is not [recognised] recognized by [the] a monoclonal antibody 4057 D2 (deposited at the CNCM under the number CNCM I-2901).

63. (Once Amended) [A] The recombinant peptide sequence according to claim 60, [comprised in the portion comprising] wherein said recombinant peptide sequence comprises all or a portion of the last 50 amino acids of the C-terminal extremity of [the sequence of Figure 10] SEQ ID No. 19, or [any] a variant thereof [of that sequence], wherein said variant is obtained by addition, substitution or deletion of one or more amino acids [retaining] and said variant retains adhesion properties .

64. (Twice Amended) [A] The peptide sequence according to claim 60, [characterized in that the] wherein said host cell is a mycobacterium.

66. (Twice Amended) A reactant for detecting an anti-HBHA antibody in a biological fluid consisting of:

a) [the] an HBHA protein purified from a preparation of mycobacterium cell walls, or a fragment thereof, determined by epitope mapping; or

b) a fragment [thereof] comprised in the last 30 to 50 amino acids in a C-terminal portion of [that] said HBHA protein, [and in particular] or in the last

50 C-terminal amino acids [shown in Figure 10, according to claim 56] of SEQ ID No. 19; or

c) a recombinant peptide sequence according to [any] claim 60.

67. (Once Amended) [A] The reactant according to claim 66, [characterized in that] wherein said recombinant peptide sequence c) is [preferably] expressed in a mycobacterium.

69. (Once Amended) [A] The kit according to claim 68, [characterized in that the] wherein said anti-antibody antibody is specific for human immunoglobulins.

70. (Once Amended) [A] The kit according to claim 68, [in which the] wherein said anti-antibody antibody is directly or indirectly labelled, either using a labelling substance, or by an enzyme which emits a labelling signal [by virtue of a] through the transformation of its substrate.

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VOTRE REFERENCE :

NOTRE REFERENCE :

I *Florence LAZARD*

Of *Ernest GUTMANN-Yves PLASSERAUD S.A.*
3 rue Chauveau-Lagarde
75008 PARIS (FRANCE)

declare that I am conversant with the French and English languages and that to the best of my knowledge and belief the following is a true translation of the facsimile letter dated June 25, 2002, from Mr. Georges Wagener, responsible at the Collection Nationale de Cultures de Microorganismes (Institut Pasteur, Paris, France) for the exercise of the duties of an International Depository Authority under the Budapest Treaty.

Signature _____

Paris, July 2, 2002

SOCIETE ANONYME
AU CAPITAL DE 500 000 €
RCS PARIS B 332 417 500
APE 741 A

ENGLISH TRANSLATION OF THE FACSIMILE LETTER DATED JUNE 25, 2002 FROM MR. GEORGES WAGENER

Madam,

Further to Mr. Franco Dante Menozzi's request, we are confirming that we have received today twelve frozen samples for each of the hybridomas identified hereunder, in view of two initial deposits under Rule 6.1 of the Budapest Treaty.

Hybridomas to deposit were registered at the CNCM on **June 25, 2002.**

Identification Reference	Registration Number
3921 E4	CNCM **I-2900**
4057 D2	CNCM **I-2901**

If a deposit is accepted, the serial number given by the CNCM is identical to the registration number and the deposit date is the registration date.

Remaining at your disposal,

Yours Sincerely,

Georges WAGENER

CNCM
Collection Nationale de Cultures de Microorganismes
INSTITUT PASTEUR
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F-75724 PARIS CEDEX 15

Tel : +33 1 45 68 82 50
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Paris, le 25 juin 2002

Cabinet GUTMANN-PLASSERAUD
67, Boulevard Haussmann
75008 Paris

A l'attention de
Madame Florence LAZARD

Par télécopie - 1 page : 01 42 66 08 90 / 03 20 87 11 58

N/R : CNCM-10976.06/02

Obj : Enregistrement de 2 hybridomes à déposer sous le Traité de Budapest

C/C : **Dr F.D. MENOZZI et Dr C. LOCHT**
Mécanismes Moléculaires de la Pathogénie Bactérienne
INSERM U447 - Institut Pasteur de Lille
1, rue du Pr Calmette - 59019 Lille Cedex

Madame,

Suite à la demande de Monsieur Franco Dante Menozzi, nous vous confirmons avoir reçu aujourd'hui en vue de deux dépôts initiaux en vertu de la règle 6.1 du Traité de Budapest douze échantillons congelés pour chacun des hybridomes identifiés ci-après.

Les hybridomes à déposer ont été enregistrés à la CNCM le **25 juin 2002**

Référence d'Identification	Numéro d'enregistrement
3921 E4	CNCM **I-2900**
4057 D2	CNCM **I-2901**

Si un dépôt est accepté, le numéro d'ordre attribué par la CNCM est identique au numéro d'enregistrement et la date du dépôt est la date de l'enregistrement.

Restant à votre disposition,
je vous prie de croire, Madame, à l'assurance de ma considération distinguée.

Georges WAGENER

EGYP

ERNEST GUTMANN - YVES PLASSERAUD S.A.
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VOTRE REFERENCE :

NOTRE REFERENCE :

I *Florence LAZARD*

Of *Ernest GUTMANN-Yves PLASSERAUD S.A.*
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75008 PARIS (FRANCE)

declare that I am conversant with the French and English languages and that to the best of my knowledge and belief the following is a true translation of the text of French Patent FR 9606168 filed on May 17, 1996.

Signature _____

Paris, July 2, 2002

SOCIETE ANONYME
AU CAPITAL DE 500 000 €
RCS PARIS B 332 417 500
APE 741 A

IDENTIFICATION AND CLONING OF A MYCOBACTERIAL ANTIGEN CORRESPONDING TO A HEPARIN-BINDING HAEMAGGLUTININ

The invention relates to peptide sequences enabling mycobacteria to adhere to host cells, in particular to epithelial cells. More particularly, the invention relates to a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen obtained from *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis*. The invention also relates to a recombinant peptide sequence enabling mycobacteria to adhere to host cells. In particular, the invention relates to the expression product of an *Escherichia coli* strain transformed with a nucleotide sequence coding for a protein enabling mycobacteria to adhere to host cells. These polypeptides can be used in immunogenic compositions, to prepare vaccines against mycobacterial infections, and for serological diagnosis of mycobacterial infections.

The invention also relates to a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells, and in particular a nucleotide sequence coding for a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen. The invention also relates to recombinant vectors comprising said nucleotide sequence and to the use of these vectors in producing recombinant host cells which can be used in therapy, in particular in anti-cancer therapy.

Mycobacteria are among the most important pathogenic micro-organisms which cause disease in both man and in animals. Mycobacterial infections are still among the main causes of death in the world. Human tuberculosis, caused by *Mycobacterium tuberculosis*, by itself leads to approximately 3 million deaths per annum (1, 2). *Mycobacterium bovis* causes tuberculosis in cattle, but it is also highly virulent in man. Leprosy, caused by *Mycobacterium leprae*, remains a major unresolved health problem in developing countries (3).

Infections by members of the *Mycobacterium avium intracellulare* complex cause disease in birds and in pigs and are among the most frequent opportunistic infections found in patients suffering from acquired

immunodeficiency syndrome (AIDS) (4, 5). Further, the recent dramatic re-appearance of tuberculosis in developed countries and the appearance and propagation of drug resistant *M. tuberculosis* strains (6) underline the difficulty of controlling mycobacterial diseases.

Molecular characterisation of the various steps in the pathogenesis of mycobacterial diseases is fundamental to the development of optimised and rational therapeutic and prophylactic approaches to such diseases. The virulence carriers are often good antigens which can be used as candidates for vaccines. Despite the importance of mycobacterial infections, little is known about the basic molecular mechanisms involved in their pathogenesis (7).

One of the initial and crucial events in bacterial pathogenesis is adhesion of the micro-organism to its target cells. Mycobacteria exhibit tropism for pulmonary macrophages (8). However, since such micro-organisms are readily transmitted by aerosol, the first structures in the host which they encounter during infection are those of the respiratory epithelium. As a result, interactions with epithelial cells or with the extracellular matrix (ECM) during the initial and subsequent steps of pathogenesis can be important (9), although they have not yet been studied to any great extent.

Within the context of the present invention, the inventors have obtained a novel mycobacterial antigen involved in adhesion of mycobacteria to epithelial type host cells. It is a 28 kDa heparin binding haemagglutinin (HBHA) which has been obtained from culture supernatants prepared from cell walls of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis.* Immunoblot analysis using polyclonal and monoclonal antibodies have indicated that HBHA differs from proteins of the antigen 85 complex and represents a novel antigen. From this basic protein, the inventors have been able to evaluate and propose the development of a series of polypeptides which can be used for diagnosis, therapy and prophylaxis.

The invention thus provides a peptide sequence enabling mycobacteria to adhere to host cells, in particular epithelial cells. More particularly, the peptide sequence of the invention is characterized in that it is a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen, in particular an antigen obtained from *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis.*

In a preferred embodiment of the present invention, the peptide sequence is characterized in that it comprises the sequence corresponding to the sequence shown in Figure 10, or any variant of that sequence which enables mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of the sequence of Figure 10.

Preferably, the invention more particularly provides a peptide sequence comprising a region involved in interactions with sulphated glycoconjugates and in heparin binding. This peptide sequence is as follows:

LRERAEETRTDTRSRVEESRARLTKLQEDLPEQLTELR.

The regions that are particularly utilized are situated at the beginning, in the middle and at the end of the chain, and contain between 7 and 15 amino acids.

The invention also concerns a peptide sequence comprising the C-terminal portion of the sequence of Figure 10, and more particularly the sequences comprising approximately the last 10 to 50 amino acids of the C-terminal portion of the sequence of Figure 10. This region of the sequence of Figure 10 is involved in the mechanism of anchoring of the protein on the bacterium, although a shorter sequence, in particular of about 10 to 20 amino acids, is sufficient to permit the anchoring of the protein.

The inventors have also expressed the nucleotide sequence coding for the peptide sequence of the invention in *E. coli.* The polypeptide obtained has a lower molecular weight than that of the purified protein

from *M. bovis* BCG or *M. tuberculosis*. These differences are the result of post-translational modifications which do not occur in *E. coli*.

The invention thus also relates to a recombinant peptide sequence, characterized in that it enables mycobacteria to adhere to host cells. More particularly, the recombinant sequence of the present invention is the expression product of a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells, in particular an antigenic sequence obtained from *M. bovis* BCG or *M. tuberculosis*, said recombinant sequence being, for example, the expression product of an *E. coli* strain transformed with a suitable nucleotide sequence.

The invention also relates to the use of one of the peptide sequences described above, whether recombinant or non recombinant, for serological diagnosis of the presence of mycobacteria. The invention also provides an immunogen composition characterized in that it comprises one of the peptide sequences described above and to the use of that peptide sequence to prepare vaccines against mycobacterial infections, particularly infections caused by *M. bovis* or *M. tuberculosis*.

The inventors have also discovered that adhesion of mycobacteria to epithelial cells can be specifically inhibited by sulphated glucides. The invention thus concerns the use of a sulphated glucide to inhibit adhesion of mycobacteria to epithelial cells. Sulphated glucides of particular interest include heparin, chondroitin sulphate and dextran sulphate as well as their synthetic derivatives.

The inventors have also isolated the whole of the gene coding for HBHA from the DNA of *M. bovis* BCG. The invention thus provides a nucleotide sequence, characterized in that it codes for a peptide sequence enabling mycobacteria to adhere to host cells. More particularly, the nucleotide sequence of the invention codes for a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen, in particular the peptide sequence of Figure 10 or any portion of that peptide sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids from said peptide sequence.

The invention also provides a recombinant host cell, characterized in that it comprises one of the nucleotide sequences described above in its genome. In one preferred embodiment of the invention, the recombinant host cell is BCG, but not exclusively, for which expression vectors directly usable for developing recombinant BCG for use in man or animal have been developed.

BCG is used in therapy, more particularly in anti-cancer therapy, in particular against superficial cancers of the bladder. In this type of therapeutic application, a correlation between the adhesion ability of the BCG and its anti-tumoral power appears to exist. Within the context of the present invention, identification of the HBHA and cloning of its gene render possible an increase in adhesion capacity via overexpression of the gene coding for HBHA.

The present invention will now be described with reference to the following Figures:

- Figure 1 shows the effect of sulphated and non sulphated glucides on mycobacterial adhesion to CHO cells and to macrophages;
- Figure 2 shows data demonstrating purification of a heparin binding protein of *M. bovis* BCG;
- Figure 3 shows a comparison of the heparin binding protein of *M. bovis* with the antigen 85 complex;
- Figure 4 shows the effect of sulphated glucides and non sulphated glucides on haemagglutinination induced by HBHA;
- Figure 5 shows the inhibition of adhesion of BCG to CHO cells by anti-HBHA antibodies;
- Figure 6 shows immunoblot analyses carried out with tuberculous anti-sera;
- Figure 7 shows the nucleotide sequence and the amino acid sequence of a fragment of HBHA deduced from a PCR fragment of chromosomal BCG DNA;
- Figure 8 shows a Southern blot analysis of chromosomal BCG DNA;

- Figure 9 shows the sequencing strategy for the gene coding for HBHA;
- Figure 10 shows the DNA sequence of the BCG gene coding for HBHA;
- Figure 11 shows a polyacrylamide gel electrophoresis and immunoblot analysis of the expression of HBHA in *E. coli*.

DETAILED DESCRIPTION OF THE INVENTION

Inhibition of mycobacterial adhesion to epithelial cells by sulphated glucides

The invention concerns the use of sulphated glucides to inhibit adhesion of mycobacteria to epithelial cells. To demonstrate that mycobacteria can adhere via sulphated glucides, the inventors tested whether soluble sulphated polysaccharides were capable of reducing the adhesion of *M. bovis* BCG to epithelial cells.

M. bovis BCG (strain 1173P2, OMS, Stockholm, Sweden, passes 3 to 8) in exponential growth were labelled by cultivating the mycobacteria for three days in Sauton medium containing 5 µCi/ml of [6-^{3}H]uracil (New England Nuclear, 24 Ci/mmole). The mycobacteria were then harvested by centrifugation (3000 x g for 5 minutes), washed twice with a Dulbecco saline phosphate buffer (DPBS) and taken up into suspension in RPMI 1640 culture medium containing 300 mg/l of L-glutamine (GIBCO), free of foetal calf serum (RPMI).

The day before the adhesion test, the wells of 24-well tissue culture plates (Nunclon, Nunc, Denmark) were inoculated with 10^5 extemporaneously cultivated cells from the ovaries of Chinese hamsters (CHO) (see Figures 1A and 1B, grey bars) or J774A.1 macrophages (ATCC TIB67) (see Figure 1B, black bars) taken up into suspension in 2 ml of RPMI with an added 10% (v/v) of decomplemented foetal calf serum (RPMI-FCS). Just before the test, the cells were washed three times with 2 ml of RPMI, and 1 ml of the mycobacterial suspension in RPMI was added to each well to obtain an infection multiplicity of 10 bacteria per eukaryote cell.

The adhesion test was carried out in the presence of increasing concentrations of D(+)galactose (Sigma) (see Figure 1A, black circles) or heparin from pork intestinal mucosa (M_r 6 kDa, Sigma) (see Figure 1A, white circles), or with 20 µg/ml of the indicated glucides (Sigma) (see Figure 1B). After 6 hours of incubation at 37°C in an atmosphere containing 5% of CO_2, the cells were washed three times in 2 ml of DPBS, and finally lysed by adding 1 ml of distilled water containing 0.1% (w/v) of sodium deoxychlorate. The radioactivity associated with the cellular lysates was counted using a liquid scintillation counter (Beckman, model LS 6000SC). Residual adhesion was expressed as a percentage of the radioactive counts per minute with respect to the counts obtained in the absence of glucide. The data shown in Figures 1A and 1B represent the averages of four experiments, and show the error bars.

As shown in Figure 1A, low concentrations of heparin substantially inhibited adhesion to CHO cells of BCG labelled with [^{3}H]uracil, while up to 100 µg/ml of galactose had no significant effect. Dextran sulphate, fucoidane and chondroitin sulphate could reduce adhesion, but no significant inhibition was observed with non sulphated mannose or dextran, even at the highest concentrations tested (1 mg/ml).

These results lead to the proposal that mycobacteria have a major sulphated glucide binding adhesin on their surface. However, as adhesion inhibition did not exceed 70%, other components are very probably involved in this process. Interestingly, interactions of BCG with J774A.1 macrophages were not affected by sulphated glucides, nor by non sulphated sugars (Figure 1B), even at concentrations of up to 1 mg/ml (results not shown). This is in agreement with previous reports naming receptors of the CR1, CR3 and CR4 complement as mycobacterial ligands on the surface of sanguine monocytes and alveolar macrophages.

Purification of a heparin binding protein present in *M. bovis* BCG and *M. tuberculosis*

The invention concerns a peptide sequence enabling mycobacteria to adhere to host cells. Inhibition of adhesion of BCG to epithelial cells by

sulphated sugars would already suggest that mycobacteria express an adhesin which interacts with sulphated glycoconjugates, such as sulphated glycoproteins or glycolipids present on the surface of the host cells. To identify the adhesins in question, BCG culture supernatants were fractionated by chromatography on heparin-sepharose.

M. bovis BCG were cultivated at 37°C in static cultures in 175 cm^3 Roux flasks (Falcon, Becton-Dickinson) containing approximately 150 ml of Sauton medium. In the stationary phase, the cultures were centrifuged (10,000 x g for 20 minutes), and 500 ml of supernatant was loaded onto a heparin-sepharose CL-6B column (1 x 5 cm) (Pharmacia) equilibrated with DPBS. The column was then washed with 100 ml of DPBS, and the retained molecules were eluted by a linear gradient of NaCl, 0-500 mM, in 100 ml of DPBS. The flow rate during all of the steps was kept at 1.5 ml/min, and the absorbance of the eluent, which was harvested in 1 ml fractions, was continuously monitored at 280 nm. These fractions were analysed by SDS-PAGE using a 12% gel followed by staining with Coomassie Brilliant Blue R-250.

The fractions analysed after the start of the gradient are shown in tracks 1-8 of Figure 2. The right hand tracks show the heparin binding protein purified from a preparation of *M. bovis* BCG cell walls. The size of the markers M_r, expressed in kDa, are given in the right hand margin.

As shown in Figure 2, a single protein of 28 kDa was eluted at approximately 350 mM of NaCl. The interaction between this protein and heparin was dependent on the sugar sulphatation as that could also be purified using dextran sulphate beads, but not with dextran beads (not shown). The same heparin binding protein was also purified from *M. tuberculosis* culture filtrates.

The production of two similar heparin binding proteins from two different mycobacterial strains confirms that proteins of this type are present in the majority of pathogenic mycobacterial strains. Homology percentages of less than 100% could lead to structural differences, although the fundamental properties of these proteins are not affected.

The invention thus also concerns heparin binding proteins with a structure related to the isolated protein and which can be obtained using its properties of adhesion to sulphated glucides. These proteins can be distinguished from the protein of the invention by additions, substitutions and deletions of amino acids without that substantially affecting their properties of adhesion to epithelial cells.

Localisation of the heparin binding mycobacterial protein on the cell surface.

In order to determine whether this protein is a strictly secreted protein or if it could be associated with the mycobacterial surface, cell wall fractions were prepared from BCG, then chromatographed on heparin-sepharose.

To prepare the cell walls, mycobacteria were cultivated in 2 litres of Sauton medium or in Long synthetic medium (Quality Biological Inc., Gaithersburg, MD) for 12 to 14 days. The bacteria were then harvested by centrifugation, washed once with DPBS containing 0.05% (v/v) of Tween 80 (DPBS/Tw), taken up into suspension in 100 ml of DPBS/Tw and heated to 80°C for 1 hour. The bacteria were centrifuged at 13,000 x g for 20 minutes, washed with DPBS/Tw, taken up into suspension in 25 ml of DPBS/Tw containing 5 nM of protease inhibitor AEBSF, RNase A and DNase I, sonically lysed intermittently for 25 minutes, then centrifuged at 13,000 x g for 20 minutes. This step was repeated and the supernatants were combined and centrifuged at 34,000 x g for 3 hours at 4°C. The residue was discarded and the final supernatant was diluted 1:2 in DPBS and chromatographed on CL-6B heparin-sepharose.

As shown in Figure 2, the 28 kDa heparin binding protein was also present in these preparations, confirming that it was associated with the surface. It was also present in cell wall fractions of *M. tuberculosis* and in tuberculine preparations (purified antigenic proteinic derivative for cutireaction) derived from *M. tuberculosis*.

Haemagglutinination activity of the mycobacterial heparin binding protein

The capacity of bacterial adhesins to agglutinate red blood cells is often used as a model to study microbial binding such as that of lectins to the receptor in eukaryotic cells. The inventors thus tested the purified heparin binding protein for its capacity to agglutinate erythrocytes.

The haemagglutinination activity of the purified heparin binding protein in culture supernatants was measured in U microtitration plates (Falcon) in the absence or in the presence of heparin (Figure 4, black squares) from pork intestinal mucosa, dextran sulphate (Figure 4, black circles) or dextran (figure 4, white circles) in concentrations of 0 to 50 µg/ml. Standard tests contained 70 µl of a fresh suspension of rabbit erythrocytes prepared in DPBS and 70 µl of purified HBHA corresponding to 1 µg of protein. The amount of haemagglutinination was read after 5 hours of incubation at room temperature. The data represent the averages over four experiments, and the error bars are shown.

Less than 0.1 µg of purified protein was able to induce haemagglutinination of rabbit erythrocytes, but not of human, sheep, goose or chicken erythrocytes (data not shown). For this reason, the inventors designated this protein as being a heparin binding haemagglutinin (HBHA). Haemagglutinination induced by HBHA was inhibited by heparin or by dextran sulphate, but not by dextran (see Figure 4), which is similar to the results obtained in the tests for adhesion of mycobacteria to CHO cells. The haemagglutinination activity was heat sensitive in that incubation of the protein for 60 minutes at 80°C destroyed haemagglutinination.

Inhibition of haemagglutinination activities and adhesion originating from mediation by HBHA, by specific polyclonal and monoclonal antibodies

Since mycobacterial adhesion to epithelial cells is inhibited by sulphated glucides, and a 28 kDa protein was purified by heparin-sepharose chromatography, it was important to establish whether mycobacterial adhesion originated from mediation by HBHA.

As a result, anti-HBHA rat polyclonal antibodies were prepared as follows: 200 µg of purified BCG HBHA underwent preparative electrophoresis on a 15% polyacrylamide gel in the presence of SDS, then electro-transferred to a nitrocellulose membrane. After rapid staining with xylidene red, the bands corresponding to HBHA were excised with care, cut into small squares and briefly sonically lysed in 1.5 ml of sterile PBS. After adding 1 ml of a monophosphoryl-lipid A solution (MPL + TDM System Adjuvant, Sigma Chemical Co., St. Louis, MO) prepared in accordance with the manufacturer's recommendations, two Fischer rats were each immunised with 1 ml of the antigen suspension. For each immunisation, 400 µl was administered intraperitoneally and two times 300 µl subcutaneously. The animals received repeat immunisations in the same manner with the same quantity of antigen one month later, and the serum was recovered 3 weeks after that repetition.

The BCG was labelled with [6-^{3}H]uracil as described above. Approximately 4 x 10^6 radiolabelled BCG were taken up into suspension in 1 ml of PBS and pre-incubated with the indicated volumes of ascetic liquids of monoclonal antibodies 3921E4 anti-HBHA (Figure 5, panel A), or with 250 µl of rat anti-HBHA polyclonal antiserum (immunitary serum, Figure 5, panel B) or naïve serum (Figure 5, control serum, panel B) for 30 minutes, at room temperature. The bacterial suspensions were then washed three times with 2 ml of DPBS to eliminate the non bound antibodies, then used in the CHO cell adhesion test in an infection multiplicity of 10, as described above.

As shown in Figure 5, adhesion of BCG to CHO cells was inhibited substantially in a dose-dependent manner by the presence of anti-HBHA antibodies. The ascites liquids containing the non pertinent monoclonal antibodies or naïve antiserums had no effect. These observations indicate that adhesion of mycobacteria to epithelial cells originates in part through the mediation of HBHA.

Immune reactivity towards HBHA by antisera from tuberculosis patients

In order to find out whether HBHA is capable of inducing an immune response in man, immuno blot analyses were carried out using purified HBHA and human antisera from tuberculosis patients. Five micrograms of the protein underwent electrophoresis using a 15% polyacrylamide-SDS gel, then were transferred to nitrocellulose membranes subsequently probed with sera diluted 100 fold originating from 7 different patients suffering from evolutive tuberculosis (see Figure 6, tracks 3A at 493) (track 1, purified HBHA, track 2, non pertinent protein), also the serum from a healthy subject (control track). The left hand tracks contained purified proteins (tracks 1 and 2) and the molecular weight markers (see PM) were stained with Coomassie blue after SDS-PAGE.

The results shown in Figure 6 show that all of the serums from tuberculosis patients contained anti-HBHA antibodies, while the serum from a healthy individual contained none of those antibodies. These results indicate that HBHA exposed on the surface is immunogenic during evolution of human tuberculosis and the presence of anti-HBHA antibodies enables the presence of mycobacterial infections to be determined.

The invention thus concerns the use of a peptide sequence or a protein of the invention, in particular one or more immunogenic regions of this sequence or protein, in the diagnosis of mycobacterial infections, in particular by showing the presence of anti-HBHA antibodies in the biological fluids. In general, a peptide sequence or a recombinant peptide sequence according to the invention, is bonded to a support and incubated with the biological fluids suspected of containing anti-HBHA antibodies. The anti-HBHA antibodies bonded to the peptide sequence of the invention are then revealed either, for example, with labelled antibodies directed against the anti-HBHA antibodies, or with non labelled antibodies directed against the anti-HBHA antibodies and antibodies labelled, for example, with a peroxidase or alkaline phosphatase type enzyme, or biotin directed against non labelled antibodies.

In detail, the complete sequence of the peptide sequence or the recombinant peptide sequence of the invention can be used but one or

more immunogenic regions of that sequence can be used which can be determined by epitope mapping techniques which are well known to the skilled person. As an example, to map the B and T epitopes present in the complete HBHA molecule, hydrophobicity profiles are used (33). Computer prediction of antigenic B cell determinants is also used (34).

The peptide sequence selected is adsorbed onto a microtitration type plate, canula, microbead or the like. The peptide sequence is bound using techniques which are known to he skilled person. Preferably, the support is a microtitration plate. The polypeptide is then diluted in a basic carbonate buffer and placed in the wells. After incubating for several hours at room temperature, a number of washes using a physiological buffer are carried out.

The peptide sequence bound to the support is then incubated with a biological fluid sample. Several types of biological fluids can be used, obtained from animals or humans. More particularly, the biological fluid can be obtained from serum, lymph, saliva or urine or it can be isolated from tissues such as cells of the lung. Incubation is carried out using the normal procedures. Here, the sera from patients are diluted and brought into contact with the peptide sequence bound to the plate. Incubation of about one hour is followed by several washes with a physiological buffer. The anti-HBHA antibodies bonded to the peptide sequence of the invention are then revealed either with the labelled antibodies directed against the anti-HBHA antibodies, or with non labelled antibodies directed against the anti-HBHA antibodies then with labelled antibodies directed against the non labelled antibodies.

The antibodies can be labelled radioactively but in general, a further normal type of labelling is preferred. Fluorescent substances such as esithiocyanatefluoriscein or enzymes such as alkaline phosphatase, peroxidase or biotin/streptavidin are routinely used labels. The choice of labelled on non labelled antibodies depends on the animal from which the biological fluids originate. If the biological fluid is human, the antibodies used are directed against human immunoglobulins.

Binding between the anti-HBHA antibodies and the labelled or non labelled antibodies is carried out using the normal techniques. As an example, the reaction takes place over one hour at room temperature and after several washes, a labelling substrate is added.

The invention also provides a kit for detecting the presence of anti-HBHA antibodies in a sample of a biological fluid. The kit comprises a peptide sequence, a recombinant peptide sequence or one or more immunogenic regions of the HBHA, adsorbed on a support and optionally, a labelled antibody (and if necessary a non labelled antibody) as well as the normal buffers and a substrate for the label.

HBHA characteristics study

Since the size of this protein is close to that of fibronectin binding proteins in the antigen 85 complex (12), Western blot analyses were used to determine whether they were related.

Purified heparin binding protein from a preparation of *M. bovis* BCG cell wall (Figure 3, track 1) or culture supernatant (track 2) was compared with purified antigen 85 complex (track 3) by immunoblot analysis (panels A, B, C) and staining with Coomassie Blue (panel D) after SDS-PAGE. Immunoblot analysis was carried out using polyclonal antibodies directed against the purified heparin binding protein (panel A), the monoclonal antibody 4057D2 (panel B) or polyclonal antibodies directed against antigen complex 85 (panel C). Tracks 1 and 2 of panels A, B and C contained 2 µg of purified protein, tracks 3 of panels A, B and C contained 7 µg of purified protein, and tracks 2 and 3 of panel D contained 4 µg and 15 µg of purified protein respectively. The size of labels M_r is shown in the margin.

Figure 3 demonstrates that polyclonal antibodies directed against the 28 kDa purified BCG heparin binding protein did not recognize purified antigen 85 complex proteins. In contrast, polyclonal and monoclonal antibodies (not shown) directed against the BCG antigen 85 complex had not succeeded in recognizing the heparin binding protein, which implies

that the proteins are distinct. This result is also supported by the different migration profiles for these proteins during SDS-PAGE (Figure 3, panel D).

The N-terminal amino acids of the purified heparin binding proteins of H37Ra *M. tuberculosis* and of BCG were sequenced. This was accomplished by subjecting 25 µg of HBHA to polyacrylamide-SDS gel electrophoresis using a 15% polyacrylamide gel. After electrophoresis, the material was transferred to a PVDF membrane (ProBlott, ABI) by electroblotting. After staining with Coomassie blue, the band corresponding to HBHA was excised and underwent automatised Edman degradation. The first 16 amino acids were Ala-Glu-Asn-Ser-Asn-Ile-Asp-Asp-Ile-Lys-Ala-Pro-Leu-Leu-Ala-Ala. The first 16 amino acids in the BCG heparin binding protein were also determined and proved to be identical to those of *M. tuberculosis*. A similarity search in protein databases showed that the heparin binding protein had not been identified before, and that it thus represents a novel mycobacterial protein. Further, the first 16 amino acids did not exhibit any significant sequence similarity with other known protein sequences.

Cloning of the BCG gene coding for HBHA

To clone the gene coding for HBHA, firstly the N-terminal sequences of internal HBHA fragments were determined. To this end, purified HBHA underwent electrophoresis as described above. After electrophoresis, the protein was digested with trypsin inside the gel. The resulting peptides were then isolated by reverse phase HPLC, and then underwent Edman degradation. Four peptides enabled the sequence to be determined:

peptide S1441:	Lys-Ala-Glu-Gly-Tyr-Leu-Glu-Ala-Ala-Thr
peptide S1443:	Xxx-Glu-Gly-Tyr-Val-Asp-Gln-Ala-Val-Glu-Leu-Thr-Gln-Glu-Ala-Leu-Gly-Lys
peptide S1446:	Xxx-Gln-Glu-Xxx-Leu-Pro-Glu-Xxx-Leu

peptide S1447: Phe-Thr-Ala-Glu-Glu-Leu-Arg

The sequence of two pairs of oligonucleotides was derived from the internal HBHA peptide sequences. The generally high G+C content in the mycobacterial DNA has led the inventors to favour G or C in the third position of the codons (wobble). The first pair of oligonucleotides originated from the S1441 peptide and had the following sequences: 5'AAG GC(G/C) GAG GG(G/C) TAC CT 3' (oligo S1441) and 5' AGG TA (G/C) CCC TC(G/C) GCC TT 3' (reverse oligo S1441). The second pair of oligonucleotides originated from the S1443 peptide and had the following sequences: 5'GAC CAG GC(G/C) GT (G/C) GAG CT 3' (oligo S1443) and 5' AGC TC (G/C) AC(G/C) GCC TGG TC 3' (reverse oligo S1443).

The chromosomal BCG DNA was extracted as described by Kremer et al (23). Polymerisation chain reactions (PCR) using 50 ng of chromosomal BCG DNA and 1 µg of either reverse S1441 and S1443, or reverse oligo S1443 and S1441, were carried out at a hybridization temperature of 50°C with 30 PCR cycles. Only the PCR carried out with the reverse S1441 and S1443 oligonucleotides produced a specific amplified DNA fragment of approximately 150 bp. This amplified fragment was again observed when the hybridization temperature was increased to 57°C.

The amplified fragment was inserted into the *Hin*dII site of pUC18 (Boehringer, Mannheim) and introduced into *Escherichia coli* XL1-Blue (New England Biolabs). The amount of recombinant *E. coli* plasmid was then analyzed using standard methods (24) and the plasmid containing the expected fragment was designated pClone5.

After purification by chromatography on a Nucleobond AX column (Macherey-Nagel) following the instructions of the supplier, a bicatenary DNA fragment of approximately 150 bp was sequenced using the dideoxyribonucleotide chain elongation termination method using [alpha-^{35}S]dCTP (1,000 Ci/mmole; Amersham) and the T7 sequencing kit (Pharmacia) following the instructions of the manufacturer. The sequence

obtained is shown in Figure 7 in which the sequence of two oligonucleotides used for the PCR is underlined. The sequence of amino acids deduced from the DNA sequence was found to correspond to the sequence of amino acids determined for peptides S1441 and S1443. This indicates that the amplified PCR fragment corresponded to an internal portion of the BCG gene coding for HBHA.

In order to clone the whole of the gene coding for HBHA, the 150 bp fragment of pClone5 was excised by digestion with restriction enzymes *Bam*HI and *Hin*dII. The fragment was then purified by electrophoresis on a 7% polyacrylamide gel and excised from the gel by electro-elution. The purified fragment was labelled with digoxigenin using the DIG (Boehringer) DNA detection and labelling kit as recommended by the manufacturer. The labelled fragment was then used in Southern Blot experiments to probe the chromosomal BCG DNA digested with *Bam*HI (Figure 8, track 1), *Eco*RI (Figure 8, track 2), *Pst*I (Figure 8, track 3), *Sma*I (Figure 8, track 4), *Acc*I (Figure 8, track 5), *Nco*I (Figure 8, track 6), *Not*I (Figure 8, track 7), *Sac*I (Figure 8, track 8) or *Sph*I (Figure 8, track 9), which underwent electrophoresis on agarose and was transferred to a nylon membrane. The membrane was then probed with the approximately 150 bp BamHI/HindII fragment from pClone5. The marker sizes are shown in the right hand margin. Southern blot analyses were carried out using standard protocols (24). As shown in Figure 8, digestion with chromosomal BCG DNA resulted in a unique fragment of about 2.5 kb which hybridized with the probe.

2.3 to 2.7 kb *Sph*I restriction fragments from chromosomal BCG DNA were then purified by preparative electrophoresis and electro-elution, and inserted in the *Sph*I site of pUC18. Recombinant plasmids were used to transform *E. coli* XL1-Blue. White colonies cultivated on gelose LB (24) with added ampicillin (150 µg/ml), isopropyl-thiogalactopyrannoside (IPTG, 40 µg/ml) and X-gal (40 µg/ml) were analyzed by hybridization on colonies using the probe labelled with digoxigenin. Of approximately 300 colonies which were analyzed, one had hybridized with the probe. Restriction

analysis of the plasmid isolated from these clones had indicated that it contained a *Sph*I fragment of 2.5 kb which had hybridized with the probe in Southern blot.

Analysis of the sequence of the BCG gene coding for HBHA

The gene coding for HBHA contained in the 2.5 kb *Sph*I fragment was sequenced using the dideoxyribonucleotide chain elongation termination method described above. The synthetic oligonucleotide sequences used for sequencing are shown in Table 1, and the sequencing strategy is shown in Figure 9. The cloned 2.5 kb *Sph*I fragment is shown by the black line. The dotted lines represent the DNA vector. The bold arrows represent the open reading frame of HBHA. The fine arrows indicate the direction and length of the DNA fragment sequenced for each oligonucleotide indicated above its respective arrow.

TABLE I: Oligonucleotides used for sequencing the gene coding for HBHA.

Name of oligonucleotide	Sequence
HBHA Seq1	5'AGC CGG TAC AAC GAG CTG GTC 3'
HBHA Seq1inv	5'GAC CAG CTC GTT GTA CCG GCT 3'
HBHA Seq2	5'CAT CCA ACA CGT CGA CTC C 3'
HBHA Seq3	5'TTG ATG TCA TCA ATG TTC G 3'
HBHA Seq4	5'CGT GGA CCA GGC GGT GGA G 3'
HBHA Seq5	5'GAC GAT CAG GAG GTT TCC CCG 3'
Reverse primer	5'AGC GGA TAA CAA TTT CAC ACA GGA 3'

The whole of the gene coding for HBHA was localized inside the 2.5 kb *Sph*I fragment, at one end of the fragment, and it was completely sequenced from two strands.

The nucleotide sequence and the derived protein sequence are shown in Figure 10. The open reading frame is shown between nucleotides 331 and 927. The first 16 codons after the optional start codon ATG 331/333 are translated into an amino acid sequence which is identical to that determined after N-terminal sequencing of the purified

HBHA protein. Upstream of the optional start codon, at residues 331-333, a stop codon TAG is found in phase at positions 310-312. This strongly suggests that the HBHA open reading frame contains no sequences coding for the conventional signal peptide, and 331/333 thus probably represents the start codon. This is supported by the presence of a putative ribosome binding site AGGAA which is found 10 to 6 nucleotides upstream of the start codon.

The deduced protein sequence exhibits a calculated molecular weight of 21331, which is lower than the apparent molecular weight estimated by SDS-PAGE. The predicted protein is composed of 198 amino acids and does not contain any cysteine, methionine, histidine or tryptophan. The five peptide sequences which have been determined were found in the predicted protein sequence of HBHA (underlined in fig. 10). Homology searches in the protein databases confirmed that HBHA is a mycobacterial protein which has not yet been identified.

The inventors have identified a region of the protein implicated in the interactions with sulphated glycoconjugates. This region is comprised in the following sequence:

LRERAEETRTDTRSRVEESRARLTKLQEDLPEQLTELR.

The peptides involved at least partially in the interactions are the following:

LRERAEE

TASRVEESRARL

EQLTELR.

The repetitions at the C-terminal extremity of the protein, rich in prolines, alanines, and lysines, account for the difference between the apparent and calculated molecular weights of the protein which undergoes post-translational modifications such as glycosylation. These repetitions are totally or partially involved in the definition of a region permitting the anchoring of the protein in the mycobacterial cell wall.

The invention thus concerns more particularly the C-terminal part of the sequence of Figure 10, and yet more particularly, the sequence comprising approximately the 10 to 50 last amino acids between amino

acids 150 and 199 of the sequence of figure 8. More preferably, the invention relates to the C-terminal end of the protein of figure 10, and more particularly, the C-terminal end comprising the repetitions rich in proline, alanine and lysine, i.e., the 50 last amino acids of the sequence of figure 8.

The invention also concerns any variant of the sequences described above obtained by addition, substitution or deletion of one or more amino acids without substantially modifying the properties of the region of interest. Among the envisaged modifications are silent mutations in the nucleotide sequences which do not modify the peptide sequence of the protein or the fragment of interest, also conservative mutations which consist of substituting one or more amino acids with the same functional characteristics as the amino acid of the native sequence. Additions or deletions of amino acids without substantially modifying the properties of the regions of interest also form part of the invention. Certain residues can be eliminated or modified by expressing a modified gene in this way in *E. coli* as described for the complete gene. If this protein binds heparin with the same affinity as whole HBHA, this signifies that the repeat sequences are not involved in that binding. In contrast, if the modified protein loses the capacity to interact with heparin, the modified region of the protein plays a role in that interaction.

Expression of the BCG HBHA gene in *Escherichia coli*

The invention also concerns a recombinant host cell, characterized in that it comprises one of the nucleotide sequences described above in its genome. More particularly, the invention concerns the transformation of host cells such as *E. coli* with one of the nucleotide sequences of the invention to produce all or part of the HBHA protein. The bacterial strain to be transformed can comprise the complete sequence coding for HBHA appearing in Figure 10 or the sequence which may be involved in interactions with sulphated glycoconjugates or the C-terminal portion of that sequence. By way of example, the complete gene coding for BCG HBHA was introduced and expressed in *E. coli*.

In order to produce BCG HBHA in *Escherichia coli*, the plasmid derived from pUC18 which contained the 2.5 kb *Sph*I fragment comprising the BCG HBHA gene was simultaneously restricted with *Nco*I and *Kpn*I. The 705 base pair fragment from this double restriction was purified by electro-elution after migration in a 1% agarose gel using standard procedures (24) and finally cloned in the expression vector pKK388-1 (Clontech, Palo Alto, Ca., UA) previously restricted with *Nco*I and *Kpn*I. The recombinant plasmid was then introduced into *E. coli* XL1-Blue using conventional transformation techniques (24).

Phenotype analysis of the strain carrying this expression plasmid was carried out by cultivating it in a liquid LB medium and stimulating the production of recombinant HBHA by adding isopropylthio-galctopyranoside (IPTG) using standard methods (24). Practically, two cultures in 500 ml erlenmeyer flasks each containing 100 ml of liquid LB medium supplemented with ampicillin in an amount of 150 µg/ml were respectively inoculated with 4 ml of *E. coli* XL1-Blue preculture containing the HBHA expression plasmid or plasmid pKK388-1 representing the control culture. The growth in the two cultures was monitored by measuring the optical density at 600 nm. When this reached 0.6, IPTG was added to the cultures in an amount of 1 mM final and culture was continued for 4 hours. Culture samples were removed before adding the IPTG and at the end of the 4 hours of culture in the presence of an inducer for analysis by polyacrylamide gel electrophoresis and by immunoblotting the production of recombinant HBHA.

The results of these different analyses are shown in Figure 11.

Tracks A and B represent total lysates before introducing *E. coli* XL1-Blue respectively transformed with pKK388-1 and the derivative of pKK388-1 containing the gene coding for HBHA. Tracks C and D represent total lysates after IPTG induction of *E. coli* XL1-Blue respectively transformed with pKK388-1 and the derivative of pKK388-1 containing the gene coding for HBHA. Tracks E contained 1 µg of purified HBHA from BCG walls. From left to right, the different panels respectively represent

staining of the polyacrylamide gel with Coomassie blue R-250 and immunoblots probed with a control murine serum, a murine serum directed against BCG HBHA, the murine monoclonal antibody 3921E4 and the murine monoclonal antibody 4057D2. The reference molecular weights are noted to the left of the panel showing staining of the polyacrylamide gel with Coomassie blue.

Analysis of the polyacrylamide gel stained with Coomassie blue shows the synthesis of a polypeptide of about 25 kDa in *E. coli* XL-1 Blue carrying the HBHA expression vector and derepressed by IPTG. This polypeptide, recognized in immunoblotting by a murine antiserum directed against the purified HBHA protein of a BCG culture supernatant, was not produced by *E. coli* XL-1 Blue carrying the pKK388-1 vector with no insert, showing that its synthesis depends on the cloned BCG DNA sequence in the expression vector. As this is also the complete sequence coding for the BCG HBHA, it was surprising to observe that the recombinant polypeptide had an apparent molecular weight which was lower than that of the purified BCG HBHA and which was 28 kDa in the gel system under consideration.

The invention thus also concerns a recombinant peptide sequence characterized in that it enables mycobacteria to adhere to host cells. More particularly, the invention concerns a peptide sequence comprising a polypeptide of about 25 kDa recognized by the monoclonal antibody 3921E4 and not recognized by the monoclonal antibody 4057D2. Preferably, the recombinant peptide sequence of the invention is the expression product of a strain of *E. coli* transformed with one of the nucleotide sequences described above and coding for a peptide sequence enabling mycobacteria to adhere to host cells, more particularly a nucleotide sequence obtained from *M. bovis* BCG or *M. tuberculosis*. The invention also concerns any variant of this recombinant peptide sequence obtained by addition, substitution or deletion of one or more nucleotides such that the transformed strain produces a different peptide sequence but

which possesses the property of adhering to host cells, and more particularly to epithelial cells.

Since the molecular weight of the HBHA deduced from the gene sequence is significantly lower than its apparent molecular weight observed after electrophoretic migration and that difference is the result of post-translational modifications of HBHA, these modifications did not occur in *E. coli*. This conclusion is supported by the fact that we observed a differential immuno-reactivity for recombinant HBHA with the two monoclonal antibodies 3921E4 and 4057D2. While the two monoclonal antibodies recognize purified BCG HBHA in an equivalent manner on immunoblotting, only 3921E4 is immunoreactive with recombinant HBHA produced in *E. coli*. This observation firstly shows that the epitopes of these two monoclonal antibodies are different and that the epitope recognized by 4057D2 on HBHA is no longer present when the latter is produced in *E. coli*, confirms that it is localized on a molecular element resulting from a post-translational modification not carried out in *E. coli*.

This post-translational modification is a glycosylation since it has already been demonstrated that the saccharide portion of a glycoprotein can be immunogenic. The saccharide moiety is highly immunogenic since immunoblot analysis using anti-HBHA murine serum gives rise to a weaker signal with recombinant HBHA than with that observed with purified BCG HBHA. This observation is all the more telling since the quantity of recombinant HBHA used in this analysis is much larger than that of natural HBHA, as shown by the Coomassie blue staining of the polyacrylamide gel. Further, since the immunoreactivity of 3921E4 is close to that observed with murine serum, the epitope recognized by this monoclonal antibody is thus partially dependent on the suspected post-translational modification.

Identification of the heparin binding site of HBHA

As it has been demonstrated that HBHA plays a direct role in the interaction between BCG and epithelial cells, an interaction which can be

inhibited by heparin, it is important to confirm that this region is involved in the interaction of BCG with epithelial cells via sulphated polysaccharides.

Overexpression of HBHA in BCG

The invention also relates to a recombinant cell host characterized in that it comprises in its genome a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells and in that this nucleotide sequence is overexpressed by said host. The term "overexpressed" used in the context of the present invention means that the recombinant host cell, and particularly a recombinant BCG, expresses a gene and produces a recombinant protein in a more important manner than the microorganism having this gene naturally.

BCG is used in anticancer therapy, in particular against superficial cancers of the bladder (27). In this therapeutic application, a correlation between the adhesion ability of the BCG and its antitumor power appears to exist (28). An increase in the adhesion capacity of BCG can thus lead to a more efficient antitumor action. The identification of HBHA and the cloning of its gene make possible an increase in the adhesion ability via overexpression of the gene encoding HBHA.

Expression vectors usable for BCG are available. In particular, these expression vectors confer resistance to mercury (31) as a single selection marker and do not thus confer antibiotic resistance, which permits to avoid that these plasmids, the replication of which is possible in several species of mycobacteria, induce antibiotic resistances to important pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Other expression vectors can also be used for the development of recombinant BCG such as vectors comprising promoters of the *hps60* gene which were described by Stoxer et al (32). These vectors are directly usable for the development of recombinant BCG, usable in man or animal. Furthermore, promoters of the 85A antigen (31) or the one of *hsp60* gene (32) can be used to express recombinant genes in BCG *in vivo*.

Expression signals of 85A antigen or of *hsp60* gene are genetically fused to the gene encoding HBHA and the whole is introduced in a shuttle vector previously described. Furthermore, HBHA gene under the control of its own promoter can also be used. BCG is transformed with these recombinant plasmids and analyzed by immunoblotting for overexpression of HBHA gene. Adhesion experiments, as described above, permit to evaluate adhesion of these recombinant BCG and to compare to non-recombinant strains on a series of epithelia, including in particular the human vesical epithelium.

The expression system in BCG is also used to study the role of regions of interest in HBHA in the mycobacteria. Truncated genes in the C-terminal region are expressed in BCG, in order to determine which positions of the C-terminal region are involved in the secretion or in the anchoring of the protein at the mycobacterial cell wall level. Other deletions or punctual mutations permit to better characterize residues involved in the post-translational modification. Indeed, expression of mutated genes can be analyzed with anti-HBHA polyclonal antibodies and the post-translational modification can be highlighted thanks to monoclonal antibody 4057D2. Indeed, reactivity with polyclonal antibodies indicates expression of the native or mutated gene, and lack of reactivity with the monoclonal antibody 4057D2 indicates lack of post-translational modification of this protein in BCG expressing a mutated gene. Accurate determination of the mutation permits to accurately confirm the region, and even the amino acid of HBHA, necessary for the post-translational modification.

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CLAIMS

1. A peptide sequence enabling mycobacteria to adhere to host cells, in particular epithelial cells.

2. The peptide sequence according to claim 1, characterized in that said sequence comprises a mycobacterial heparin-binding haemagglutinin (HBHA) type antigen, in particular an antigen obtained from *M. bovis* BCG or *M. tuberculosis*.

3. The peptide sequence according to claim 1 or 2, characterized in that said sequence comprises the peptide sequence of Figure 10, or any variant of said sequence which enables mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said sequence.

4. The peptide sequence according to claim 3, characterized in that it comprises the C-terminal portion of the peptide sequence of Figure 10, more particularly the sequence comprising the last 50 amino acids of the peptide sequence of Figure 10 or any variant of that sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said sequence.

5. The peptide sequence according to any one of claims 1 to 4, characterized in that it comprises the following peptide sequence:
LRERAEETRTDTRSRVEESRARLTKLQEDLPEQLTELR
or any portion or variant of that sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said peptide sequence.

6. The peptide sequence according to any one of claims 1 to 5, characterized in that it is recognized by the monoclonal antibody 4057D2.

7. A recombinant peptide sequence, characterized in that it enables

mycobacteria to adhere to host cells, in particular epithelial cells.

8. The recombinant peptide sequence according to claim 7, characterized in that said sequence is the expression product of a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells, more particularly a peptide sequence obtained from *M. bovis* BCG or *M. tuberculosis*.

9. The recombinant peptide sequence according to claim 8, characterized in that said sequence is the expression product of a strain of *E. coli* transformed with a nucleotide sequence coding for a peptide sequence enabling mycobacteria to adhere to host cells, more particular a nucleotide sequence obtained from *M. bovis* BCG or *M. tuberculosis*.

10. The recombinant peptide sequence according to claim 8, characterized in that it comprises a recombinant polypeptide of about 25 kDa recognized by the monoclonal antibody 3921E4 and not recognized by the monoclonal antibody 4057D2.

11. The recombinant peptide sequence according to any one of claims 7 to 10, characterized in that it comprises the expression product of a nucleotide sequence coding for the peptide sequence of Figure 10 or for any portion of said peptide sequence enabling mycobacteria to adhere to host cells.

12. The recombinant peptide sequence according to any one of claims 7 to 10, characterized in that it is the expression product of the nucleotide sequence coding for the C-terminal portion, more particularly the sequence comprising the last 50 amino acids of the peptide sequence of Figure 10 or for any portion of said peptide sequence enabling mycobacteria to adhere to host cells.

13. Use of a peptide sequence according to any one of claims 1 to 12, to prepare vaccines against mycobacterial infections, particularly

infections caused by *M. bovis* or *M. tuberculosis*.

14. Use of a peptide sequence according to any one of claims 1 to 12, for diagnosis of a mycobacterial infection.

15. The use according to claim 14, characterized in that said peptide is used to detect anti-HBHA antibodies in a biological fluid.

16. An immunogenic composition, characterized in that it comprises a peptide sequence according to any one of claims 1 to 12.

17. Use of sulphated glucides or sulphated glycoconjugates to inhibit the adhesion of mycobacteria to epithelial cells.

18. The use according to claim 17, characterized in that the sulphated glucide is selected from the group comprising in particular heparin, chondroitin sulphate and dextran sulphate as well as their natural or synthetic derivatives.

19. A nucleotide sequence characterized in that it codes for a peptide sequence enabling mycobacteria to adhere to host cells, in particular to epithelial cells.

20. The nucleotide sequence according to claim 19, characterized in that said sequence codes for a mycobacterial heparin-binding haemagglutinin type (HBHA) antigen.

21. The nucleotide sequence according to claim 19 or 20, characterized in that said sequence codes for a peptide sequence comprising the peptide sequence of Figure 10 or any portion of said peptide sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said peptide sequence.

22. The nucleotide sequence according to claim 21, characterized in that said sequence codes for a peptide sequence corresponding to the C-terminal portion, more particularly the sequence comprising the last 50 amino acids of the peptide sequence of Figure 10 or any

variant of said sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said peptide sequence.

23. The nucleotide sequence according to any one of claims 19 to 22, characterized in that said sequence comprises a sequence which codes for the following peptide sequence:
LRERAEETRTDTRSRVEESRARLTKLQEDLPEQLTELR
or any variant of said peptide sequence enabling mycobacteria to adhere to host cells and obtained by addition, substitution or deletion of one or more amino acids of said peptide sequence.

24. A recombinant vector, characterized in that it comprises a nucleotide sequence according to any one of claims 19 to 23.

25. A recombinant host cell, characterized in that it comprises a nucleotide according to any one of claims 19 to 23 in its genome.

26. The recombinant host cell according to claim 25, characterized in that said host is a mycobacterium, in particular BCG.

27. The host according to claim 25 or 26, characterized in that said nucleotide sequence is overexpressed by said host.

28. Use of a recombinant host cell according to any one of claims 25 to 27, for anti-cancer therapy.

29. A kit for detecting the presence of anti-HBHA antibodies in a sample of a biological fluid, said kit comprising a peptide sequence according to any one of claims 1 to 12, or one or more immunogenic regions of said sequence adsorbed on a support and optionally, a labelled antibody (and if necessary a non labelled antibody) as well as the usual buffers and a label substrate.

ABSTRACT

The invention concerns peptide sequences enabling mycobacteria to adhere to host cells, in particular epithelial cells. More particularly, the invention concerns a mycobacterial heparin-binding haemagglutinin type (HBHA) antigen obtained from *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis*. The invention concerns also a recombinant peptide sequence enabling mycobacteria to adhere to host cells. The invention relates in particular to the expression product of a strain of *Escherichia coli* transformed with a nucleotide sequence encoding a protein that enables mycobacteria to adhere to host cells. These peptide sequences can be used in immunogenic compositions, to prepare vaccines against mycobacterial infections, and for serologic diagnosis of mycobacterial infections.

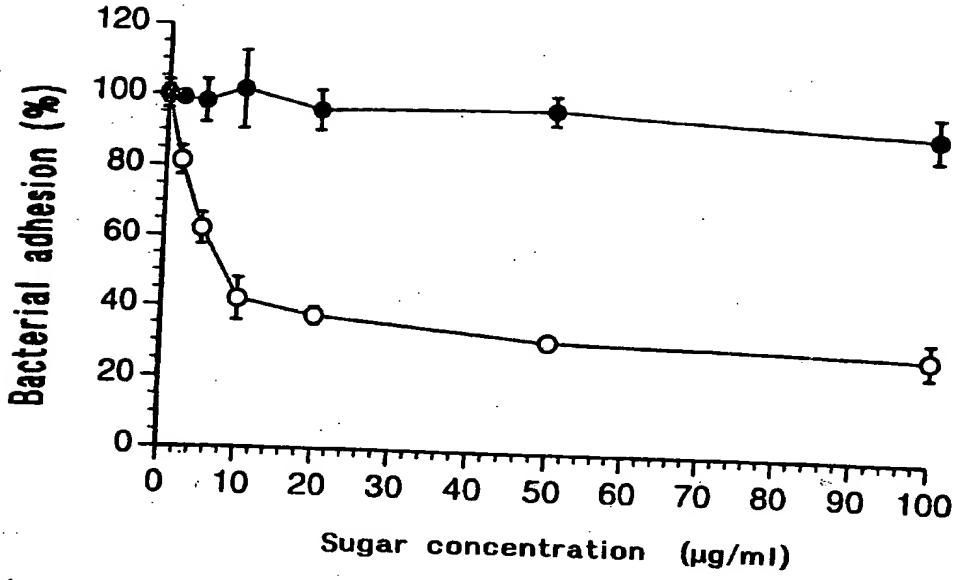

Bacterial adhesion (%)
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70 80 90 100
Sugar concentration (µg/ml)

Fig. 1A

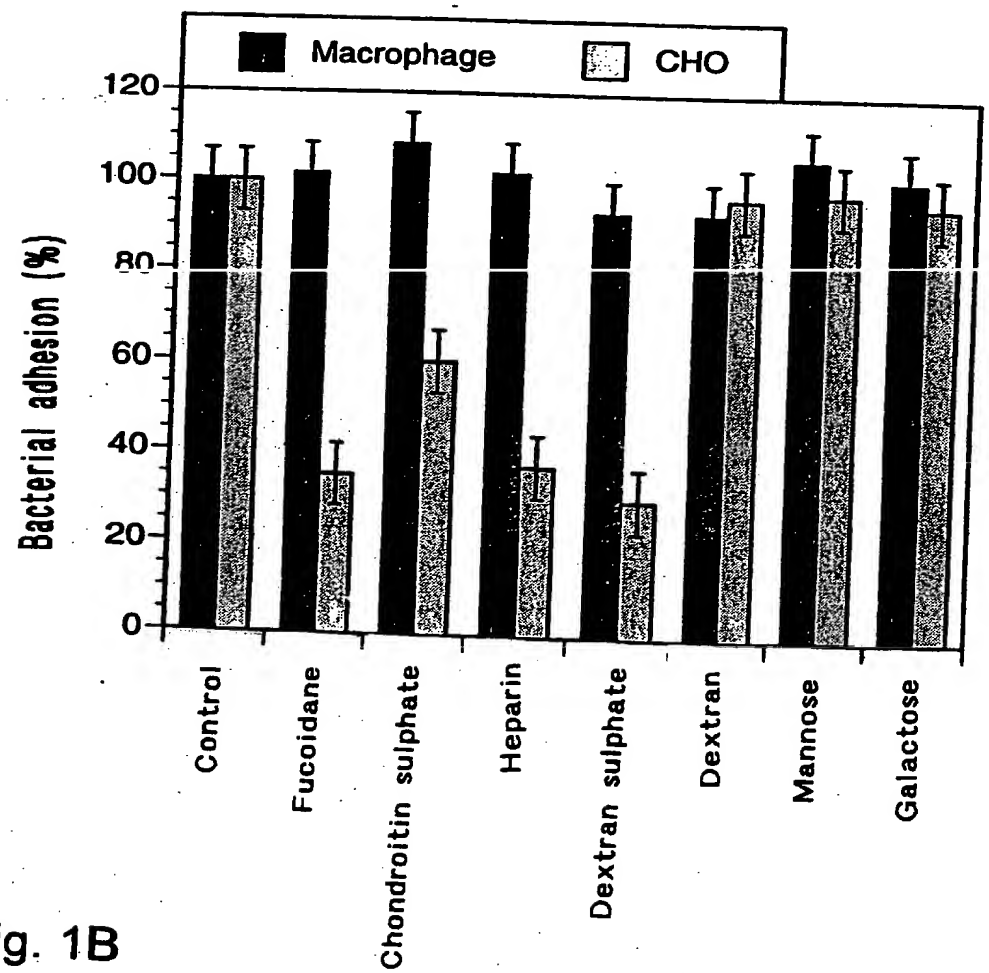

Macrophage
CHO
Bacterial adhesion (%)
120
100
80
60
40
20
0
Control
Fucoidane
Chondroitin sulphate
Heparin
Dextran sulphate
Dextran
Mannose
Galactose

Fig. 1B

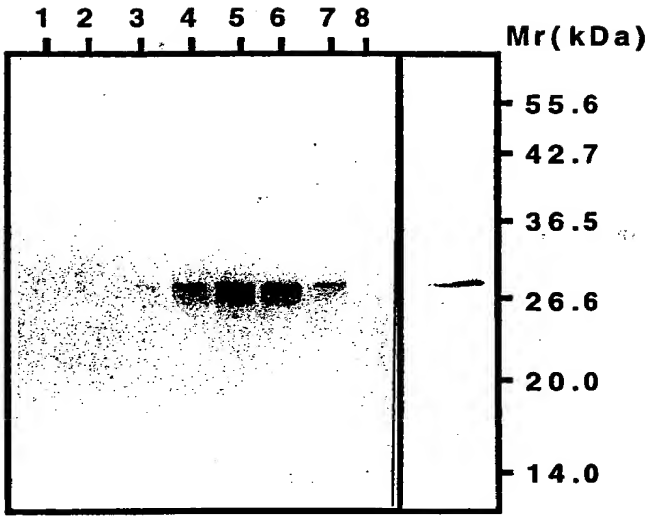


Fig. 2

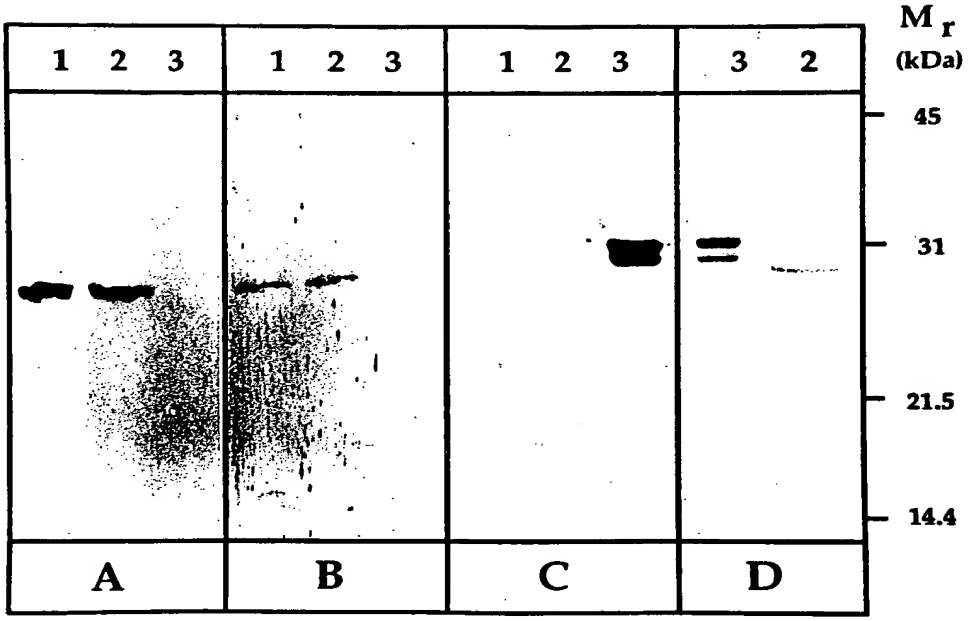


Fig. 3

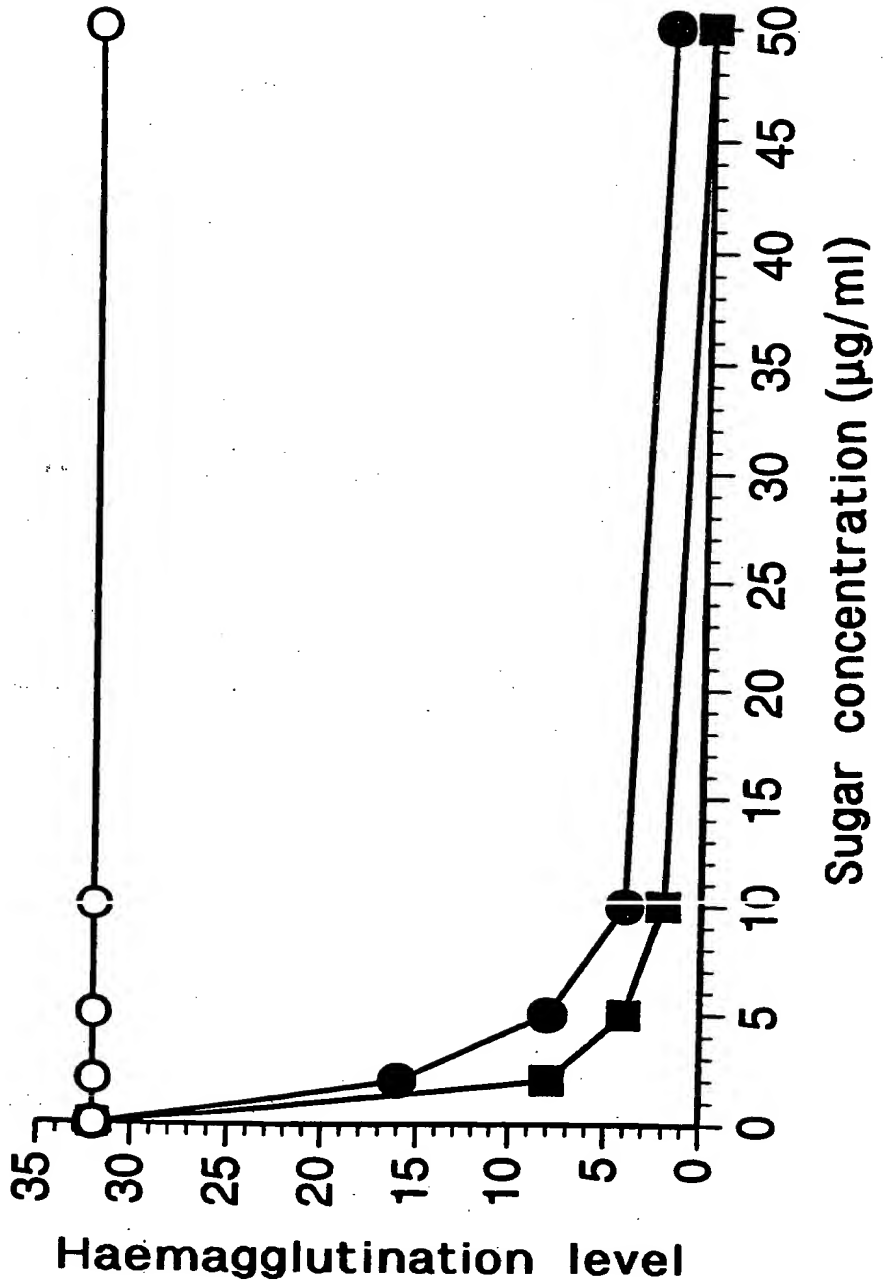

Sugar concentration (µg/ml)
0
5
10
15
20
25
30
35
40
45
50
Haemagglutination level
35
30
25
20
15
10
5
0

Fig. 4

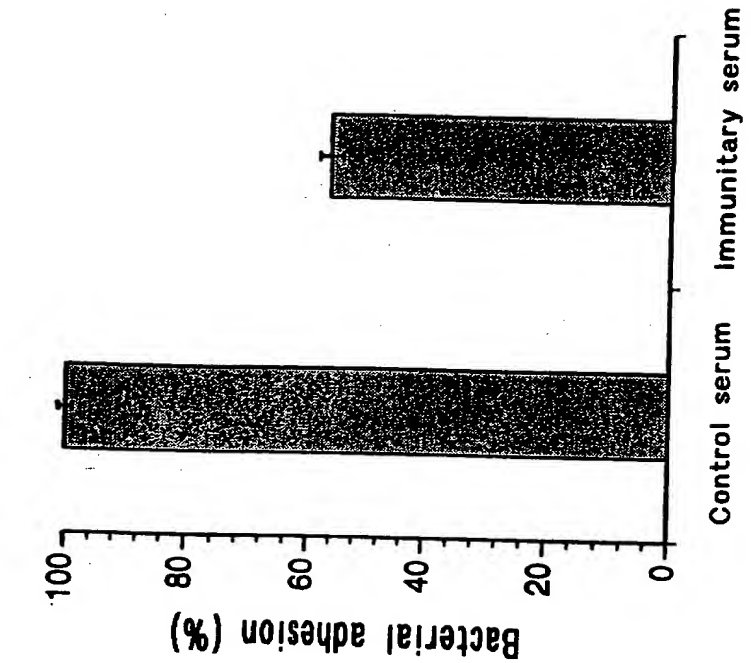

Bacterial adhesion (%)
100
80
60
40
20
0
Control serum
Immunitary serum

Fig. 5B

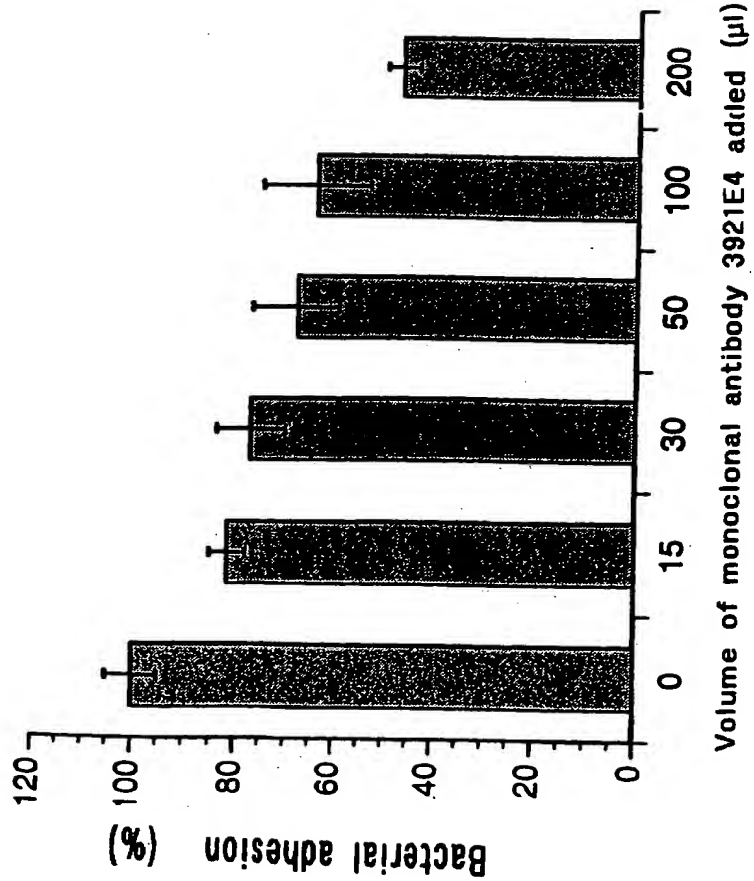

Bacterial adhesion (%)
120
100
80
60
40
20
0
0
15
30
50
100
200
Volume of monoclonal antibody 3921E4 added (µl)

Fig. 5A

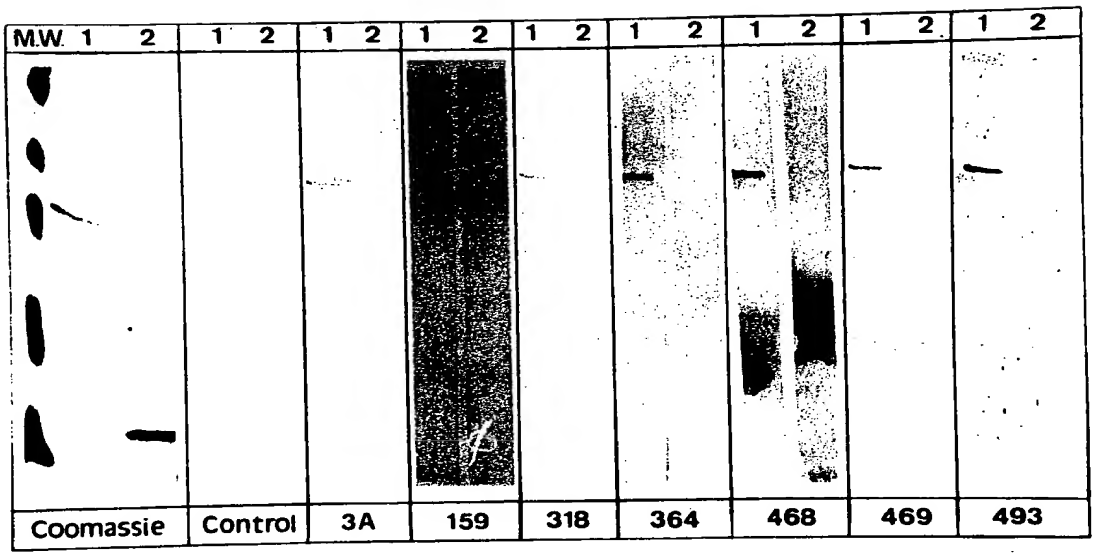

M.W. 1 2
1 2
1 2
1 2
1 2
1 2
1 2
1 2
1 2
Coomassie
Control
3A
159
318
364
468
469
493

Fig. 6

K A E G Y L E A A T S R Y N E L V
aag gcc gag ggc tac ctc gag gcc gcg act agc cgg tac aac gag ctg gtc
ttc cgg ctc ccg atg gag ctc cgg cgc tga tcg gcc atg ttg ctc gac cag
oligo 1441

E R G E A A L E R L R S Q Q S F E
gag cgc ggt gag gcc gct cta gag cgg ctg cgc agc cag cag agc ttc gag
ctc gcg cca ctc cgg cga gat ctc gcc gac gcg tcg gtc gtc tcg aag ctc

E V S A P A E G Y V D Q A V E L
gaa gtg tcg gcg ccc gcc gaa ggc tac gtg gac cag gcg gtc gag ct
ctt cac agc cgc ggg cgg ctt ccg atg cac ctg gtc cag cgc ctc ga
oligo 1443 rev.

Fig. 7

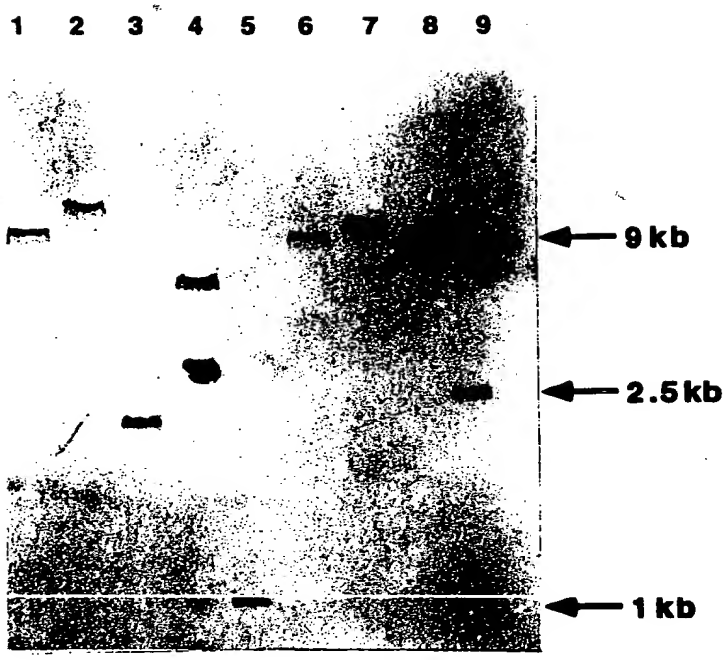
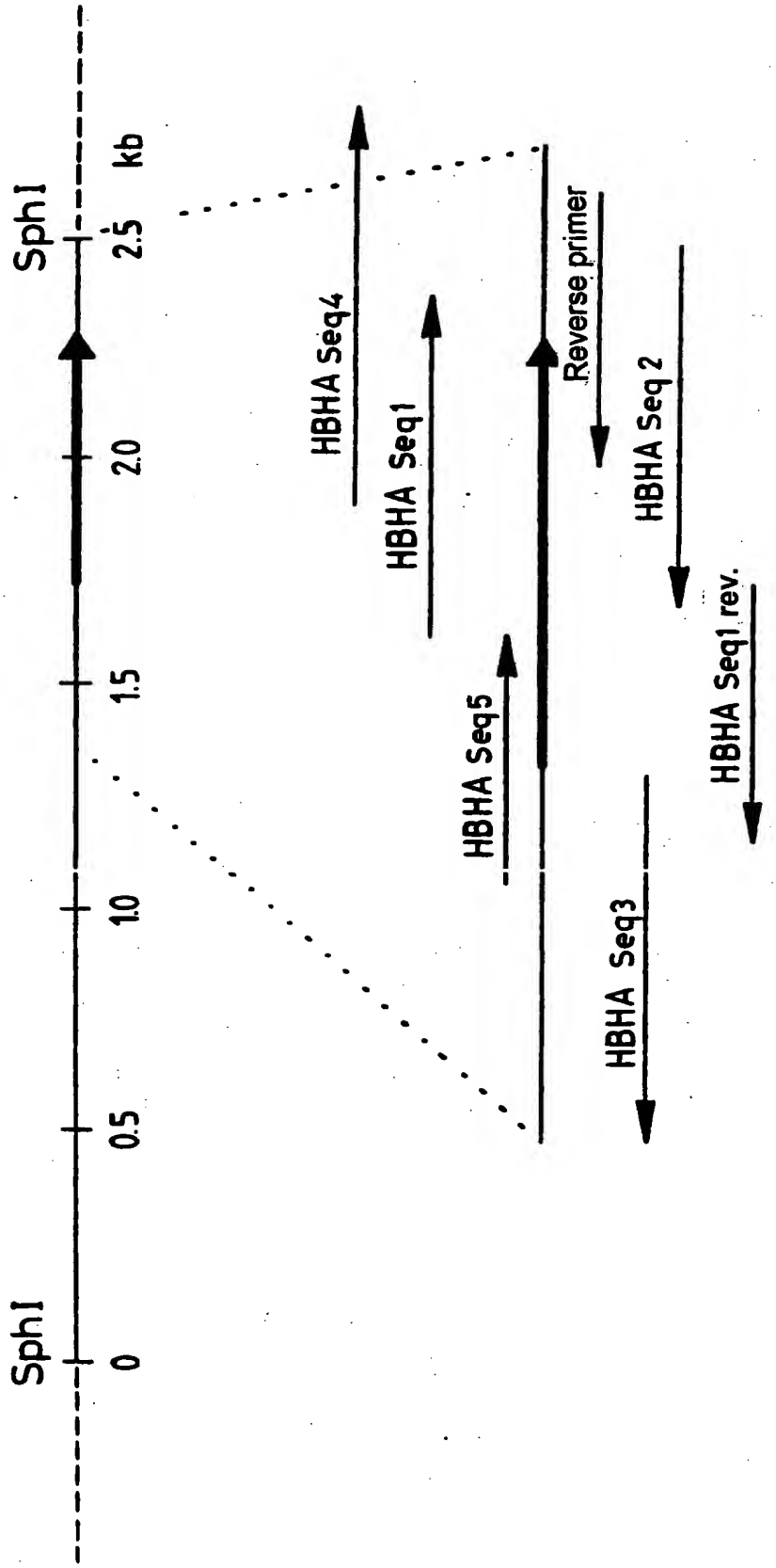


Fig. 8

FIG_9


SphI
0
0.5
1.0
1.5
2.0
2.5
kb
SphI
HBHA Seq4
HBHA Seq1
HBHA Seq5
Reverse primer
HBHA Seq 2
HBHA Seq1 rev.
HBHA Seq3

```
1
cgg ctg gcg ggt aat caa acc tga agg aca gtc atc tgg gtg agg tcg acc gca ggc tga

61
tcc agc cga tcg gcc ggc gct ggc caa cag cga ctc cgt cga tga cgt gca gca aag gag

121
aca tgt agt gac cgg atc agc tgg gcc tga cat cta cga act cga ccg aca acc gac ccg

181
acg atc agg agg ttt ccc cgg caa gtc gcg tgc cat gtc aat ccg cgg gtc ttg act agt

241
cct ccc tgg agg agc cga cgc ttg ccc caa cgt cca gac caa aga tgt aag aac gcc gat

301
atc aga aaa tag tta atg aaa gga ata ccc atg gct gaa aac tcg aac att gat gac atc
                                    M   A   E   N   S   N   I   D   D   I
361
aag gct ccg ttg ctt gcc gcg ctt gga gcg gcc gac ctg gcc ttg gcc act gtc aac gag
K   A   P   L   L   A   A   L   G   A   A   D   L   A   L   A   T   V   N   E

421
ttg atc acg aac ctg cgt gag cgt gcg gag gag act cgt acg gac acc cgc agc cgg gtc
L   I   T   N   L   R   E   R   A   E   E   T   R   T   D   T   R   S   R   V

481
gag gag agc cgt gct cgc ctg acc aag ctg cag gaa gat ctg ccc gag cag ctc acc gag
E   E   S   R   A   R   L   T   K   L   Q   E   D   L   P   E   Q   L   T   E

541
ctg cgt gag aag ttc acc gcc gag gag ctg cgt aag gcc gcc gag ggc tac ctc gag gcc
L   R   E   K   F   T   A   E   E   L   R   K   A   A   E   G   Y   L   E   A

601
gcg act agc cgg tac aac gag ctg gtc gag cgc ggt gag gcc gct cta gag cgg ctg cgc
A   T   S   R   Y   N   E   L   V   E   R   G   E   A   A   L   E   R   L   R

661
agc cag cag agc ttc gag gaa gtg tcg gcg ccc gcc gaa ggc tac gtg gac cag gcg gtg
S   Q   Q   S   F   E   E   V   S   A   P   A   E   G   Y   V   D   Q   A   V

721
gag ttg acc cag gag gcg ttg ggt acg gtc gca tcg cag acc cgc gcg gtc ggt gag cgt
E   L   T   Q   E   A   L   G   T   V   A   S   Q   T   R   A   V   G   E   R

781
gcc gcc aag ctg gtc ggc atc gag ctg cct aag aag gct gct ccg gcc aag aag gcc gct
A   A   K   L   V   G   I   E   L   P   K   K   A   A   P   A   K   K   A   A

841
ccg gcc aag aag gcc gct ccg gcc aag aag gcg gcg gcc aag aag gcg ccc gcg aag aag
P   A   K   K   A   A   P   A   K   K   A   A   A   K   K   A   P   A   K   K

901
gcg gcg gcc aag aag gtc acc cag aag tag tcg ggc tcc gaa tca cca tcg act ccg agt
A   A   A   K   K   V   T   Q   K   *

961
cgc cca cgg ggc gac tcg gag tcg acg tgt tgg atg caa acc gca tag tct gaa tgc gtg

1021
agc cac ctc gtg ggt acc gtc atg ctg gta ttg ctg gtc gcc gtc ttg gtg aca gcg gtg

1081
tac gcg ttt gtg cat gc
              SphI
```

Fig. 10

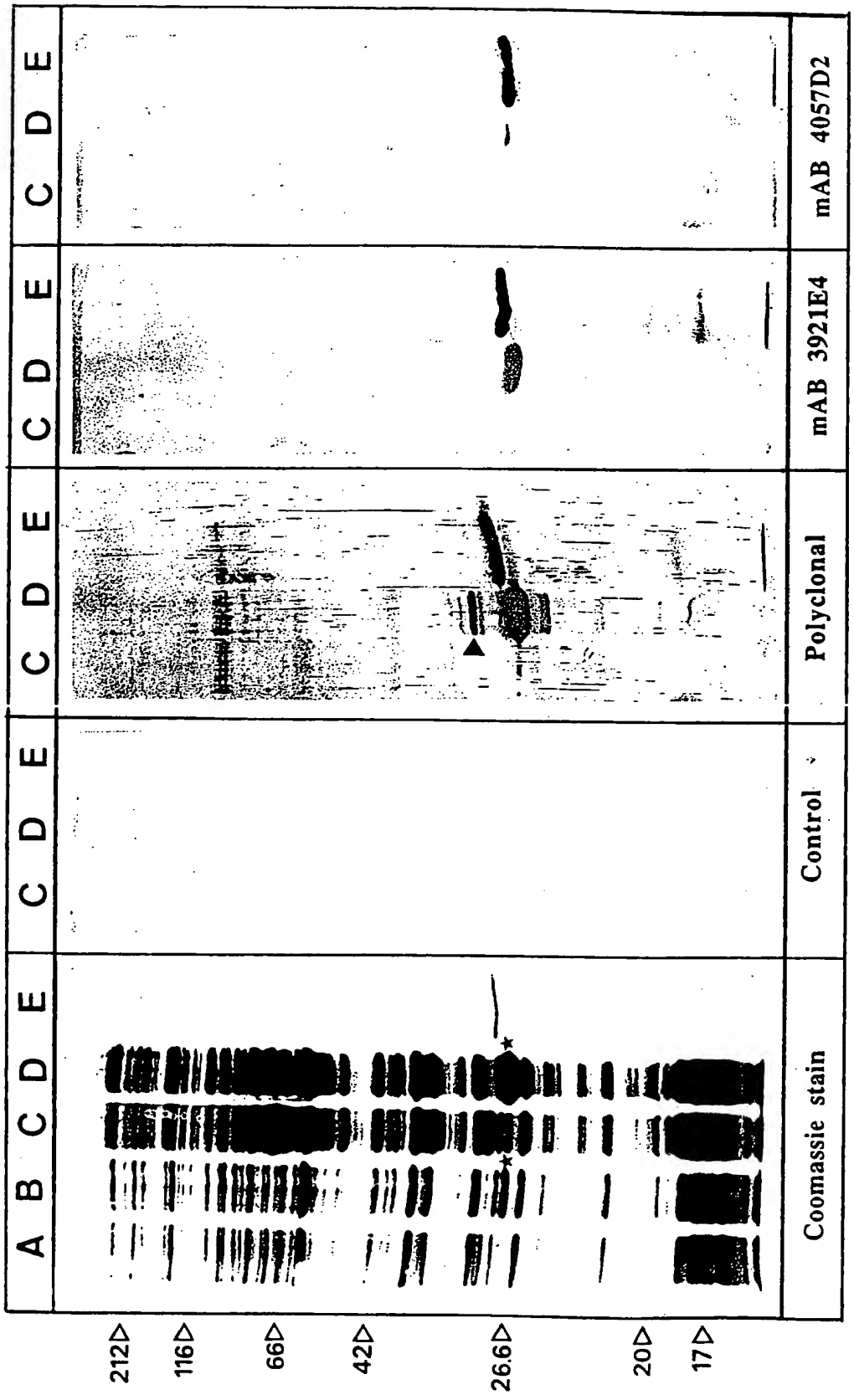

A B C D E
C D E
C D E
C D E
Coomassie stain
Control
Polyclonal
mAB 3921E4
mAB 4057D2
212
116
66
42
26.6
20
17

Fig. 11